

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION



WO 9603438A1

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> :		(11) International Publication Number: WO 96/03438			
C07K 16/28, C12N 5/20, G01N 33/577, 33/68, A61K 39/395		A1	(43) International Publication Date: 8 February 1996 (08.02.96)		
(21) International Application Number:	PCT/US95/09458	(81) Designated States:	AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).		
(22) International Filing Date:	26 July 1995 (26.07.95)	(30) Priority Data:	26 July 1994 (26.07.94) US		
(71) Applicant:	AMGEN INC. [US/US]; Amgen Center, 1840 Dehavilland Drive, Thousand Oaks, CA 91320-1789 (US).	(72) Inventor:	ELLIOTT, Steven, G.; 1040 Golden Crest Avenue, Newbury Park, CA 92649 (US).		
(74) Agents:	ODRE, Steven, M. et al.; Amgen Inc., Amgen Center, 1840 Dehavilland Drive, Thousand Oaks, CA 91320-1789 (US).	<b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>			
(54) Title: ANTIBODIES WHICH ACTIVATE AN ERYTHROPOIETIN RECEPTOR					
(57) Abstract					
Antibodies and fragments thereof which activate an erythropoietin receptor and stimulate erythropoiesis are described. Also described are hybridoma cell lines which produce the antibodies and methods and compositions for the treatment of anemia.					

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

ANTIBODIES WHICH ACTIVATE  
AN ERYTHROPOIETIN RECEPTOR

Field of the Invention

5        This invention relates to antibodies which recognize an erythropoietin receptor. More particularly, the invention relates to antibodies which activate an erythropoietin receptor and stimulate erythropoiesis.

10

Background of the Invention

Erythropoietin (EPO) is a glycoprotein hormone involved in the growth and maturation of erythroid progenitor cells into erythrocytes. EPO is produced by the liver during fetal life and by the kidney of adults and stimulates the production of red blood cells from erythroid precursors. Decreased production of EPO, which commonly occurs in adults as a result of renal failure, 20 leads to anemia. EPO has been produced by genetic engineering techniques involving expression and secretion of the protein from a host cell transfected with the gene encoding erythropoietin. Administration of recombinant EPO has been effective in the treatment of anemia. For 25 example, Eschbach et al. (N. Engl J Med 316, 73 (1987)) describe the use of EPO to correct anemia resulting from chronic renal failure.

The purification of human urinary EPO was described by Miyake et al. (J. Biol. Chem. 252, 5558 (1977)). The 30 identification, cloning, and expression of genes encoding erythropoietin is described in U. S. Patent No. 4,703,008 to Lin. A description of a method for purification of recombinant EPO from cell medium is included in U. S. Patent No. 4,667,016 to Lai et al.

- 2 -

Little is known about the mechanism by which EPO stimulates erythropoiesis. While it is clear that EPO activates cells to grow and/or differentiate by binding to specific cell surface receptors, the specific mechanism of activation as well as the structure of the receptor and any associated protein(s) is not completely understood. The erythropoietin receptor (EPO-R) is thought to exist as a multimeric complex. Sedimentation studies suggested its molecular weight is  $330\pm48$  kDa (Mayeux et al. *Eur. J. Biochem.* 194, 271 (1990)). Crosslinking studies indicated that the receptor complex consists of at least two distinct polypeptides, a 66-72 kDa species, and 85 and 100 kDa species (Mayeux et al. *J. Biol. Chem.* 266, 23380 (1991)); McCaffery et al. *J. Biol. Chem.* 264, 10507 (1991)). A distinct 95 kDa protein was also detected by immunoprecipitation of EPO receptor (Miura & Ihle *Blood* 81, 1739 (1993)). Another crosslinking study revealed three EPO containing complexes of 110, 130 and 145 kDa. The 110 and 145 kDa complexes contained EPO receptor since they could be immunoprecipitated with antibodies raised against the receptor (Miura & Ihle, *supra*). Expression of a carboxy-terminal truncated EPO receptor resulted in detection of the 110 kDa complex but not the 145 kDa complex. This suggests that the higher molecular weight complex contains polypeptides present in the 110 kDa complex and an additional 35 kDa protein.

Further insight into the structure and function of the EPO receptor complex was obtained upon cloning and expression of the mouse and human EPO receptors (D'Andrea et al. *Cell* 57, 277 (1989); Jones et al. *Blood* 76, 31 (1990); Winkelmann et al. *Blood* 76, 24 (1990); PCT Application No. WO90/08822; U.S. Patent No. 5,278,065 to D'Andrea et al.) The full-length human EPO receptor is a 483 amino acid transmembrane protein with an approximately 224 amino acid extracellular domain and

- 3 -

a 25 amino acid signal peptide. The human receptor shows about an 82% amino acid sequence homology with the mouse receptor. The cloned full length EPO receptor expressed in mammalian cells (66-72KDa) has been shown 5 to bind EPO with an affinity (100-300 nM) similar to that of the native receptor on erythroid progenitor cells. Thus this form is thought to contain the main EPO binding determinant and is referred to as the EPO receptor. The 85 and 100 KDa proteins observed as part 10 of a cross-linked complex are distinct from the EPO receptor but must be in close proximity to EPO because EPO can be crosslinked to them. The 85 and 100KDa proteins are related to each other and the 85KDa protein may be a proteolytic cleavage product of the 100KDa 15 species (Sawyer J. Biol. Chem. 264, 13343 (1989)).

A soluble (truncated) form of the EPO receptor containing only the extracellular domain has been produced and found to bind EPO with an affinity of about 1nM, or about 3 to 10-fold lower than the full-length 20 receptor (Harris et al. J. Biol. Chem. 267, 15205

(1992); Yang & Jones Blood 82, 1713 (1993)). The reason for the reduced affinity as compared to the full length protein is not known. There is a possibility that other protein species may also be part of the EPOR complex and 25 contribute to EPO binding thus increasing the affinity.

In support of this possibility is the observation of Dong & Goldwasser (Exp. Hematol. 21, 483 (1993)) that fusion of a cell line with a low affinity EPO receptor with a CHO cell which does not bind EPO resulted in a 30 hybrid cell line exhibiting high EPO binding affinity of the receptor for EPO. In addition, transfection of a full length EPOR into CHO cells resulted in a cell line with both high and low affinity receptors as measured by Scatchard analysis. Amplification of the EPOR copy 35 number increased the low affinity but not high affinity binding. These results are consistent with the presence

- 4 -

of a limited quantity of a protein present in CHO cells that converts the low affinity EPOR to high affinity.

Activation of the EPO receptor results in several biological effects. Three of the activities include

5 stimulation of proliferation, stimulation of differentiation and inhibition of apoptosis (Liboi et al. Proc. Natl. Acad. Sci. USA 90, 11351 (1993); Koury Science 248, 378 (1990)). The signal transduction pathways resulting in stimulation of proliferation and 10 stimulation of differentiation appear to be separable (Noguchi et al. Mol. Cell. Biol. 8, 2604 (1988); Patel et al. J. Biol. Chem. 267, 21300 (1992); Liboi et al. *ibid*). Some results suggest that an accessory protein may be necessary for mediating the differentiation 15 signal (Chiba et al. Nature 362, 646 (1993); Chiba et al. Proc. Natl. Acad. Sci. USA 90, 11593 (1993)). However there is controversy regarding the role of accessory proteins in differentiation since a constitutively activated form of the receptor can

20 stimulate both proliferation and differentiation (Pharr et al. Proc. Natl. Acad. Sci. USA 90, 938 (1993)).

Activation of the EPO receptor may be due to its dimerization. That is, EPO may act as a crosslinker between two EPO receptor molecules. There is evidence 25 in support of this proposal. An arginine to cysteine mutation at position 129 of the murine EPO receptor results in constitutive activation of the receptor, presumably because of a disulfide bond formed between two receptors subunits (Yoshimura et al. Nature 348, 647 30 (1990)). In addition EPOR is found in multimeric complexes in cells (Miura & Ihle Arch. Biochem. Biophys. 306, 200 (1993)). However, isolation of a stable multimeric form of purified EPO soluble receptor has not 35 been reported. In addition, dimerization of EPOR may be required, but not by itself be sufficient for complete activation of cells. For example, dimerization may

- 5 -

result in a proliferative signal but not a differentiation signal. That is, accessory proteins may be required to send the differentiation signal.

The possible relationship between EPO receptor dimerization and activation may be exploited to identify compounds which are different from EPO but activate the receptor. For example, antibodies possess two identical binding sites for antigen. An anti-EPOR antibody can bind two EPOR molecules and could bring them into close proximity to each other to allow dimerization. In order to function *in vivo*, these antibodies must recognize the EPOR on surfaces of cells and bind in a way that allows activation of the signal transduction pathway. In addition, it is desirable that activation result in both proliferation and differentiation of erythroid progenitors. A similar approach to understand the activation of human growth hormone receptor (Fuh et al. *Science* 256, 1677 (1992)) and epidermal growth factor receptor (Schreiber et al. *Proc. Natl. Acad. Sci. USA* 78, 7535 (1981)) has been reported.

It would be desirable to identify molecules which have the property of activating the EPO receptor and stimulating erythropoiesis. In order to do so, an understanding of the mechanism of EPO receptor activation and signal transduction is important. One approach to elucidating this mechanism may be to identify antibodies which recognize the EPO receptor so as to activate the receptor and stimulate erythropoiesis. Such antibodies are useful in therapeutic and diagnostic applications and would also be useful for probing EPO receptor function.

The following references describe antibodies which bind to the mouse or human EPO receptor:

D'Andrea et al. in The Biology of Hemopoiesis, Wiley-Liss, Inc. (1990) pp. 153-159, generated polyclonal anti-peptide antibodies against an amino-

- 6 -

terminal and a carboxy-terminal peptide of murine EPO receptor. The antibodies were shown to react with mouse EPO receptor in a Western blot.

Bailey et al. *Exp. Hematol.* **21**, 1535-1543 (1993)

5 generated polyclonal anti-peptide antibodies against synthetic peptides homologous to the extracellular and cytoplasmic domains of the mouse EPO receptor. Receptor activation by these antibodies, as measured by <sup>3</sup>H thymidine uptake into spleen cells from phenylhydrazine  
10 treated mice, was not detected .

Baynes et al. *Blood* **82**, 2088-2095 (1993) generated a polyclonal antibody to an amino-terminal peptide in the human EPO receptor. The antibody was shown to react with a soluble form of the receptor present in human

15 serum.

D'Andrea et al. *Blood* **82**, 46-52 (1993) generated monoclonal antibodies to human EPO receptor. The antibodies bind to Ba/F3 cells transfected with the human EPO cDNA clone and some inhibit EPO binding and

20 neutralize EPO-dependent growth.

Fisher et al. *Blood* **82**, 197A (1993) used the same monoclonal antibodies as described in D'Andrea, *supra* to distinguish erythroid progenitor cells having EPO-dependent growth and maturation from those having EPO-independent growth and maturation.

None of the antibodies described in the aforementioned references were reported to activate the EPO receptor or stimulate the growth and/or maturation of erythroid progenitor cells.

30

Therefore, it is an object of the invention to produce antibodies which recognize an EPO receptor and bind to it such that the receptor is activated. It is a further object of the invention to produce antibodies 35 which bind to an EPO receptor and stimulate erythropoiesis by stimulating the proliferation and/or

- 7 -

differentiation of erythroid progenitor cells to erythrocytes. Such antibodies are useful in the treatment of anemia or in the diagnosis of diseases characterized by dysfunctional EPO receptor. Further,  
5 such antibodies may lead to the identification of therapeutic agents for the treatment of anemia.

Summary of the Invention

10 The invention relates to antibodies or fragments thereof which activate an erythropoietin receptor. Screening of antibodies which recognize the human EPO receptor has revealed that two antibodies, designated Mab 71 and Mab 73, stimulated the proliferation of UT7-  
15 EPO cells, an EPO-dependent cell line that does not proliferate in the absence of added EPO. Further, Mab 71 stimulated erythroid colony formation from erythroid progenitors in human blood. The antibodies encompassed by the invention may recognize an epitope on an EPO  
20 receptor which is recognized by Mab 71 or Mab 73. The antibodies are preferably monoclonal antibodies and may be humanized or human antibodies. Also included are hybridoma cell lines which produce the antibodies of the invention.  
25 Also provided for are methods and kits for detecting EPO receptors in biological samples wherein the methods and kits comprise EPO receptor antibodies of the invention. Pharmaceutical compositions comprising EPO receptor antibodies and pharmaceutically acceptable  
30 adjuvants are also encompassed by the invention. Such compositions may be used to treat patients having disorders characterized by low red blood cell levels.

- 8 -

Description of the Figures

Figure 1 shows the results of an ELISA assay that measured the binding to the indicated concentrations of 5 synthetic peptides by Mab 71. The peptides correspond to the indicated amino acid residues of human EPO receptor. Residue 1 is the amino terminal proline found in secreted EPOR upon cleavage of the leader sequence.

10 Figure 2 shows the effect of varying amounts of rHuEPO protein and purified Mabs 71 and 73 on  $^3\text{H}$  thymidine uptake of UT7-EPO cells.

15 Figure 3 shows the effect of varying amounts of rHuEPO protein, Mab 71, Mab 73 or a non neutralizing control Mab directed against EPO (Mab F12) on inhibition of  $^{125}\text{I}$  EPO binding to EPO receptors on the surface of OCIM1 cells.

20 Figure 4 shows a coomassie stained SDS gel of purified preparations of monoclonal antibodies 71 and 73 as well as monoclonal antibody fragments (fabs) derived from Mabs 71 and 73. Samples were run under either reducing (plus 2-mercaptoethanol) or nonreducing (minus 25 2-mercaptoethanol) conditions.

Figure 5 shows the effect of varying amounts of purified rHuEPO protein, Mab 71 or Fab 71 on  $^3\text{H}$  thymidine uptake of UT7-EPO cells.

30 Figure 6 shows the effect of varying amounts of purified Mab 71 or Fab 71 on  $^3\text{H}$  thymidine uptake of UT7-EPO cells to which are also added 30 munits/ml of recombinant human EPO (rHuEPO).

- 9 -

Figure 7 shows a photograph of purified CD 34<sup>+</sup> cells from peripheral blood which were grown 21 days in methylcellulose in the presence of EPO or Mab 71 under serum free growth conditions. Photos are of cells 5 incubated with 500 munits/ml EPO (A), 25 munits/ml EPO (B), or 2.1 micrograms/ml Mab 71(C).

Figure 8 shows the effect of varying amounts of rHuEPO, Mab 71 and a control monoclonal antibody raised 10 to Her2/neu on the formation of erythroid colonies from erythroid precursors when grown under serum free growth conditions in soft agar.

15 Detailed Description of the Invention

Monoclonal antibodies (Mabs) which recognize the erythropoietin receptor have been generated by immunizing mice with purified soluble human EPO receptor. Soluble human EPO receptor was expressed and 20 purified as described in Examples 1 and 2. Of those Mabs which reacted with soluble human EPO receptor in enzyme-linked immunosorbent assays (ELISAs), 96 mabs were selected for further screening. These mabs were tested for EPO receptor binding by BIACore analysis 25 (Example 4A) and for binding to EPO receptor on the surface of transfected CHO cells by FACS (Example 4C). The results of these screenings are shown in Table 1. While a number of antibodies bound EPO receptor as determined by BIACore analysis, only five antibodies of 30 the 96 tested bound EPO receptor displayed on the surface of transfected CHO cells as determined by FACS scanning. 24 antibodies which were positive in ELISA assays (including those five which were positive by FACS scanning) were tested for stimulation of UT7-EPO cell 35 proliferation. Surprisingly, it was found that two

- 10 -

antibodies, designated Mab 71 and Mab 73, stimulated the uptake of  $^3\text{H}$  thymidine into a UT7-EPO cell line (Komatsu et al. *Blood* **82**, 456 (1993)) in the absence of EPO (Example 8A). The UT7-EPO cell line requires the  
5 presence of EPO in its medium for growth. Therefore, the stimulation of UT7-EPO cell growth is likely due to the activation of EPO receptor by Mab 71 and Mab 73. As shown in Figure 2, the response of UT7-EPO cells was greater in the presence of Mab 71 than Mab 73. It was  
10 further found that Mab 71 stimulated erythroid colony formation from human erythroid precursors (see Example 9). This is the first instance of an antibody stimulating the formation of erythroid colonies from erythroid precursors.

15

The invention provides for an antibody or fragment thereof which activates an erythropoietin receptor. As used herein, the term "activation of an EPO receptor" denotes one or more molecular processes which an EPO  
20 receptor undergoes that result in transduction of a signal to the interior of a receptor-bearing cell, wherein the signal ultimately brings about one or more changes in cellular physiology. Cellular responses to EPO receptor activation are typically changes in the  
25 proliferation or differentiation of receptor-bearing cells. Receptor-bearing cells are typically erythroid progenitor cells. Presently, the molecular events leading to signal transduction by EPO receptor are poorly understood. However, as indicated in the  
30 background, some evidence suggests that EPO receptor dimerization is at least one event which is likely to be required for activation. The present disclosure also provides support for this idea. As shown in Figure 5, stimulation of  $^3\text{H}$ -thymidine uptake in UT7-EPO cells by  
35 Mab 71 is abolished when substituted by the corresponding Fab fragment designated Fab 71.

- 11 -

Therefore, replacement of the intact, bivalent antibody with a corresponding monovalent fragment eliminates the proliferative response. In addition Mab 71 inhibits activation of the EPO receptor at high concentrations.

5 Both of these observations support the dimerization model of activation for the EPO receptor. Mab 71 has been shown to interact with a synthetic peptide of residues 49 to 78 of the human EPO-R (see example 6). Thus this region of EPO-R when bound by a cross linker

10 such as Mab 71 can result in activation of EPO-R. It is understood that molecules that cross-link two EPO-R molecules by binding to residues 49 to 78 are also encompassed by the invention. These molecules could be antibodies or other bivalent molecular entities that

15 have the property of crosslinking two EPO receptors by binding to residues contained within the region between residues 49 and 78 thereby resulting in dimerization and activation of the EPO receptor.

20

EPO receptors of the invention will preferably be mammalian EPO receptors and, in a particularly preferred embodiment, will be human EPO receptor. It is understood that analogs of human EPO receptors are also

25 encompassed by the invention. Such analogs are constructed by insertions, deletions, extensions or substitutions of amino acids in the human EPO receptor sequence. Examples of EPO-R analogs have been described in U.S. Patent No. 5,292,654 to Yoshimura et al. wherein

30 substitution of a cysteine residue at position 129 of the EPOR amino acid sequence resulted in constitutively activated EPOR. In general, EPO-R analogs having amino acids changes in regions other than the antibody binding domains necessary for activation wherein said analogs

35 retain secondary and tertiary structure of the human EPO receptor may be recognized by the antibodies of the

- 12 -

present invention. It has been shown that Mab 71 interacts with a synthetic peptide of residues 49 to 78 of the human EPO-R (see Example 6). Therefore, EPO-R analogs having changes in amino acid residues other than 5 those at positions 49 to 78 and retaining the human EPO receptor secondary and tertiary structure are likely to be recognized by Mab 71. The numbering of amino acid residues in the human EPOR polypeptide as used herein starts with proline at position 1, which is the amino 10 terminal residue after cleavage of the 25 amino acid signal peptide.

Antibodies of the invention bind to an epitope on an EPO receptor which is involved in receptor 15 activation. In one embodiment, antibodies recognize an epitope on an EPO receptor which is recognized by Mab 71 or an epitope which is recognized by Mab 73. Mab 71 recognizes a synthetic peptide spanning amino acid residues 49 to 78 in the human EPO-R. Therefore, it is 20 likely that Mab 71 recognizes an epitope on EPO-R which is defined in whole or in part by this sequence. As used herein, the term "epitope" refers to the region of an EPO-R bound by an antibody wherein the binding prevents association of a second antibody to an EPO-R.

25 The invention also provides polyclonal antibodies, and monoclonal antibodies and fragments thereof. Antibody fragments encompass those fragments which activate an EPO receptor. Also encompassed are 30 humanized antibodies, typically produced by recombinant methods, wherein human sequences comprise part or all of an antibody which activates an EPO receptor. Examples of humanized antibodies include chimeric or CDR-grafted antibodies (US Patent Nos. 4,816,567 and 5,225,539). 35 Also included are fully human antibodies to EPO receptor produced in genetically-altered mice (see PCT

- 13 -

Application No. 93/12227). Antibodies of the invention may also have a detectable label attached thereto. Such a label may be a fluorescent (e.g., fluorescein isothiocyanate, FITC), enzymatic (e.g., horseradish peroxidase), affinity (e.g., biotin) or isotopic label (e.g.,  $^{125}\text{I}$ ).

Also encompassed by the invention are hybridoma cell lines producing a monoclonal antibody which activates an EPO receptor. In one embodiment, the hybridoma cell line produces a monoclonal antibody which recognizes an epitope on an EPO receptor which is recognized by Mab 71 or Mab 73. Generation of hybridoma cell lines producing monoclonal antibodies to human EPO-R are described in Example 3. The hybridoma cell line which produces Mab 71 has been deposited with the American Type Culture Collection, Rockville, MD on July 26, 1994 under accession no. HB11689. The hybridoma cell line which produces Mab 73 has been deposited with the American Type Culture Collection, Rockville, MD on July 26, 1994 under accession no. HB11690.

The antibodies of the present invention are useful in diagnosing anemia and other diseases characterized by dysfunctional EPO-R. In one embodiment, a method of detecting in a biological sample an EPO receptor which is capable of which being activated comprising the steps of: (a) contacting the sample with an antibody which activates an EPO receptor; and (b) detecting activation of the receptor by the antibody. The biological samples include tissue specimens, intact cells, or extracts thereof. Antibodies may be used as part of a diagnostic kit to detect the presence of EPO receptors in a biological sample. Such kits employ antibodies having an attached label to allow for detection. The antibodies are useful for identifying normal or abnormal

- 14 -

receptors. The presence of abnormal receptors in a biological sample may be indicative of disorders such as Diamond Blackfan anemia, where it is believed that the EPO receptor is dysfunctional.

5       Antibodies of the invention are useful for treating disorders characterized by low red blood cell levels. Included in the invention are methods of modulating the endogenous activity of an EPO receptor in a mammal, preferably methods of increasing the activity of an EPO 10 receptor. In general, any condition treatable by erythropoietin, such as anemia, may also be treated by the antibodies of the invention. Therapeutic antibodies are administered by an amount and route of delivery that is appropriate for the nature and severity of the 15 condition being treated and may be ascertained by one skilled in the art. Preferably, administration is by injection, either subcutaneous, intramuscular, or intravenous.

20       The invention provides for a pharmaceutical composition comprising a therapeutically effective amount of an antibody which activates an EPO-R together with a pharmaceutically acceptable adjuvant, wherein the adjuvant may be selected from one or more of a diluent, carrier, preservative, emulsifier, anti-oxidant and/or 25 stabilizer. A "therapeutically effective amount" as used herein refers to that amount of antibody which provides a therapeutic effect for a given condition and administration regimen. In the present invention, the therapeutic effect is stimulation of red blood cell 30 production as evidenced by a rise in hematocrit in the patient being treated. In a preferred embodiment, the antibodies are humanized or human antibodies which may be prepared using procedures known to the skilled 35 worker. Pharmaceutically acceptable adjuvants are known to one skilled in the art and are surveyed extensively

- 15 -

in Remington's Pharmaceutical Sciences, 18th ed. A.R. Gennaro, ed. Mack, Easton, PA (1990).

5 The following examples are offered to more fully illustrate the invention, but are not construed as limiting the scope thereof.

EXAMPLE 1

10 Production of soluble human erythropoietin receptor

A. Isolation of clones for expression of soluble human erythropoietin receptor.

15 Using a clone containing the human erythropoietin receptor as described by Jones et al. supra, the PCR technique was used to obtain a clone for expression of soluble human erythropoietin receptor (sHuEPOR). Primers for PCR amplification of human erythropoietin receptor were:

20

5' primer:

CTC CAA GCT TGC CGT CAC CAT GGA CCA CCT CGG GGC GTC CCT  
(SEQ. ID NO:1); and

25 3' primer:

CAG GTC TAG ATT ACT AGG GAT CCA GGT CGC TAG GC  
(SEQ. ID NO:2)

30 PCR reactions were carried out using 2.5 ng of a plasmid containing human EPOR, 5 pmol of each of the above oligonucleotide primers, 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM Mg Cl<sub>2</sub>, 200 μM each dNTP and 1 unit of Taq polymerase. Amplification was for 5 cycles of 30 sec. at 94°C, 1 min. at 50°C, 1 min at 72°C, followed by 35 20 cycles of 30 sec. at 94°C, 1 min. at 55°C, 1 min at 72°C. DNA was purified by passage through a G-50 size

- 16 -

exclusion column (Boehringer Mannheim Corp.), then digested with Hind III and XbaI and ligated into the expression vector pDSRa2 (DeClerck et al. J. Biol. Chem. 266, 3893 (1991)) which has also been digested with Hind III and XbaI. Clones containing the desired insert were verified by DNA sequence analysis.

The d40EPOR clone was made by PCR from a full length human EPOR clone (see above). The carboxy terminus of d40EPOR is tyr467, the result of adding a stop codon within the primer. Primers for PCR amplification were:

5' primer:

5'-CTC CAA GCT TGC CGT CAC CAT GGA CCA CCT CGG GGC GTC  
15 CCT-3'  
(SEQ. ID NO:1); and

3' primer:

5'-AGG TCG ACT ACT AGT AGT CAG TTG AGA-3'  
20 (SEQ. ID NO:3)

PCR amplification used pfu polymerase in pfu buffer2 (Stratagene, La Jolla, CA). Reaction conditions were: 1 cycle at 96° for 30 sec., 45° for 1 min., 72° for 1 min.; 25 cycles at 96° for 1 min., 55° for 1 min., 72° for 2 min. A final 72° incubation for 5 min. was then performed. The reaction products were separated by agarose gel electrophoresis and the approximately 1.3 Kb band was isolated using a gene clean kit ( BIO 101, Vista, CA.). The purified fragment was ligated into PCR II (TA cloning kit, Invitrogen, San Diego, CA). Recombinants were identified by restriction analysis and sequenced to confirm the desired inserts were present. A HindIII-SalI fragment was isolated as described above 35 and ligated into an isolated pDSRa2 vector that had been previously cut with HindIII and SalI. The resultant

- 17 -

vector, pDSR $\alpha$ EPORd40 was used for expression in CHO cells.

5 B. Expression of soluble human EPOR and d40 EPOR in CHO cells

The expression plasmid pDSR $\alpha$ 2-EPOR-X contains sequences encoding human EPOR amino acids Met1-Pro249 as shown in Jones et al. supra. Plasmid pDSR $\alpha$ EPORD40 contains sequences encoding Met1-Tyr467. Ten micrograms of each plasmid were independently introduced into CHO 10 cells by calcium phosphate mediated transfection (Wigler et al. Cell 11, 233 (1977)). Individual colonies were selected based upon expression of the dihydrofolate 15 reductase gene from the vector. Expression of human EPOR was monitored by RNA hybridization (Hunt et al., Exp. Hematol, 19: 779 (1991)) and by Western immuno blotting using an affinity purified antibody. Cell lines which were positive in these assays were selected for further expansion. Cell lines were adapted to 30nM 20 Methotrexate (Mtx) to stimulate amplification of EPO-R expression.

Generation of conditioned media containing soluble human EPOR was done in both roller bottles and a hollow fiber bioreactor. Roller bottles were innoculated with 25 2 x 10<sup>7</sup> cells in 200 ml growth medium (DMEM: Ham's F12 (1:1) supplemented with non-essential amino acids (NEAA), 30nM Mtx and 5% fetal bovine serum (FBS) (reagents from GIBCO, Grand Island, NY)). Upon reaching confluence in 3-4 days, the media was replaced with 200 30 ml DMEM: Ham's F12, NEAA, 30 nM Mtx with no serum. Conditioned media was harvested after 6-7 days and replaced with fresh serum-free media. Second and third harvests were collected.

A Cell Pharm biorector cartridge was innoculated 35 with 5 X 10<sup>8</sup> cells in growth medium (as above) supplemented with 5 ug/mL gentamicin. The pH was

- 18 -

maintained at 7.3. Beginning on day 12 after inoculation the cells were weaned off of serum to generate serum-free conditioned media. Harvesting of conditioned media began on day 17.

5

#### EXAMPLE 2

##### Purification of soluble human erythropoietin receptor

10 Four different preparations of soluble recombinant human EPOR were made. In the first preparation, Epoxy-activated Sepharose 6B (Pharmacia, Piscataway, NJ) is coupled with recombinant human erythropoietin (rHuEPO) essentially as per manufacturer's instructions. 218 mg of rHuEPO in 4.5 mL of 32 mM ZnCl<sub>2</sub> is added to 7.2 g of 15 Epoxy-activated Sepharose 6 B previously hydrated and washed with H<sub>2</sub>O. This slurry is titrated to pH 10.8 then mixed overnight at room temperature. Any remaining reactive groups are then blocked by addition of 20 ethanalamine to a final concentration of 1 M and mixed for 4 hours at room temperature. The subsequent steps are performed at 8° ± 2° C. The coupled resin (Epoxy-EPO) is packed into a column and washed with alternating cycles of 0.5 M NaCl / 0.1 M HOAc pH 4 and 0.5 M NaCl / 25 0.1 M Borate pH 8. The column is equilibrated with 140 mM NaCl / 10 mM Tris pH 7.6 (TBS). It is loaded with 1560 mL of roller bottle produced conditioned media from CHO cells expressing soluble EPO-R (sHuEPO-R). After loading is complete, the column is washed with 300 mM 30 NaCl / 10 mM Tris pH 7.6 then the bound sHuEPOR is eluted with 1 M NaCl / 3 M urea / 10 mM Tris pH 7.6. Two UV<sub>280</sub> absorbing peaks elute with this buffer. The second peak to elute, which contains the sHuEPOR, is pooled and diluted 20-fold with H<sub>2</sub>O. The diluted pool 35 is then loaded to a 1 mL prepacked column of Mono Q (Pharmacia) and eluted with a NaCl gradient in 10 mM

- 19 -

Tris pH 7.6. A single peak elutes, which is pooled, aliquoted and stored frozen at -80° C.

In the second preparation, a larger Epoxy-EPO column is made. 20.4 g of Epoxy-activated Sepharose 6 B 5 is hydrated and washed with H<sub>2</sub>O, then with acetone and finally with 50% formamide in H<sub>2</sub>O pH 10.6. 729 mg of rHuEPO in 15 mL of H<sub>2</sub>O is titrated to pH 10.6, added to the resin and mixed overnight at room temperature. Any remaining reactive groups are then blocked by addition 10 of ethanolamine to a final concentration of 1 M and mixed for 140 minutes at room temperature. The subsequent steps are performed at 8° ± 2° C. The Epoxy-EPO is packed into a column and washed with 3 M urea / 750 mM NaCl / 10 mM Tris pH 7.6, the column is then 15 equilibrated with TBS. 100 mL of bioreactor produced conditioned media from CHO cells expressing sHuEPOR are mixed with 2 mL of Q Sepharose Fast Flow (Pharmacia). It is incubated for 30 minutes at 8° ± 2° C with frequent mixing, then filtered through a 0.45 micron 20 cellulose nitrate bottle top filter (Corning). The filtrate is loaded to the Epoxy-EPO column, washed with 250 mM NaCl / 10 mM Tris pH 7.6, then eluted with 3 M urea / 750 mM NaCl / 10 mM Tris pH 7.6. The eluted peak is pooled and diluted 20 fold with H<sub>2</sub>O. The diluted 25 pool is then loaded to a 15 mL column of Q Sepharose Fast Flow and eluted with a NaCl gradient in 10 mM Tris pH 7.6. The single peak that elutes is pooled, aliquoted and stored frozen at -80° C.

In the third preparation, the same Epoxy-EPO column 30 used in preparation 2 is used. 850 mL of roller bottle produced conditioned media from CHO cells expressing SEPO-R are mixed with 1.7 mL of Q Sepharose Fast Flow. It is processed in the same manner as is done in preparation 2.

35 In the fourth preparation, 7.25 L of bioreactor produced conditioned media from CHO cells expressing

- 20 -

sHuEPOR are mixed with 110 mL of Q Sepharose Fast Flow. It is incubated for 1 hour at 8° ± 2° C with frequent mixing, then filtered through a 0.45 micron cellulose nitrate bottle top filter. The filtrate is then diluted 5 with 7.25 L of H<sub>2</sub>O and loaded to a 770 mL column of Q Sepharose Fast Flow equilibrated in 20 mM Tris pH 7.6. The column is eluted with a NaCl gradient in 20 mM Tris pH 7.6. Fractions containing significant amounts of sHuEPOR based on SDS-PAGE analysis are pooled. Solid 10 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> is added to the pool to a final concentration of 1.2 M then filtered through a 0.45 micron cellulose nitrate bottle top filter. The filtrate is loaded to a 60 mL column of Phenyl Sepharose 6 (low sub, Pharmacia) and eluted with a decreasing gradient of 1.2 M to 0 M 15 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> in 20 mM Tris pH 7.6. The major eluting peak is pooled and made 2.4 M in (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> to precipitate the sHuEPORt. The precipitated sHuEPOR is harvested by centrifugation, resuspended with H<sub>2</sub>O and titrated to pH 7.9 with Tris-HCl. The resultant solution is filtered 20 through a 0.45 micron cellulose nitrate filter, aliquoted and stored frozen at -80° C.

#### EXAMPLE 3

25 Preparation and Screening of hybridoma cell lines

A. Enzyme-linked Immunosorbent Assay (EIA)

EIAs were initially performed to determine serum antibody (Ab) titres of individual animals, and later 30 for screening of potential hybridomas. Flat bottom, high-binding, 96-well microtitration EIA/RIA plates (Costar Corporation, Cambridge, MA) were coated with purified sHuEPOR at 5 µg per ml carbonate-bicarbonate buffer, pH 9.2 (0.015 M Na<sub>2</sub>CO<sub>3</sub>, 0.035 M NaHCO<sub>3</sub>). Fifty 35 µl of the Ab were added to each well. Plates were then covered with acetate film (ICN Biomedicals, Inc., Costa

- 21 -

Mesa, CA) and were incubated at room temperature (RT) on a rocking platform for 2 hours or over-night at 4°C. sHuEPOR lot #1 was used after the first and second boost, lot #2 was used after the third boost. sHuEPOR 5 lots #3 and 4 were used for screening of hybridomas. Plates were blocked for 30 minutes at RT with 250 µl per well 5% BSA solution prepared by mixing 1 part BSA diluent/blocking solution concentrate (Kirkegaard and Perry Laboratories, Inc.) with 1 part deionized water 10 (dH<sub>2</sub>O). Blocking solution having been discarded, 50 µl of serum 2-fold dilutions (1:400 through 1:51,200) or hybridoma tissue culture supernatants were added to each well. Serum diluent was 1% BSA (10% BSA diluent/blocking solution concentrate diluted 1:10 in 15 Dulbecco's Phosphate Buffered Saline, D-PBS; Gibco BRL, Grand Island, NY), while hybridoma supernatants were tested undiluted. In the case of hybridoma testing, one well was maintained as a conjugate control, and a second well as a positive Ab control. Plates were again 20 ~~incubated at RT, rocking, for 1 hour, then washed 4~~ times using a 1x preparation of wash solution 20x concentrate (Kirkegaard and Perry Laboratories, Inc.) in dH<sub>2</sub>O. Goat anti-mouse IgG heavy- and light- chain specific horseradish peroxidase conjugated secondary Ab 25 (Boehringer Mannheim Biochemicals, Indianapolis, IN) diluted 1:1000 in 1% BSA was then incubated in each well for 30 minutes. Plates were washed as before, blotted dry and ABTS Peroxidase single component substrate (Kirkegaard and Perry Laboratories, Inc.) was added. 30 Absorbance was read at 405 nm for each well using a Microplate EL310 reader (Bio-tek Instruments, Inc., Winooski, VT). Half-maximal titre of serum antibody was calculated by plotting the log<sub>10</sub> of the serum dilution versus the optical density at 405 nm, then extrapolating 35 at the 50% point of the maximal optical density obtained by that serum. Hybridomas were selected as positive if

- 22 -

optical density scored greater than 5-fold above background.

B. Immunization

5        Ten, 4.5 week old Balb/c mice (Charles Rivers Laboratories, Wilmington, MA) were subcutaneously injected (SQI) with 50 µg sHuEPOR; lot 1; antigen) emulsified in Complete Freund's Adjuvant (CFA; 50% vol/vol; Difco Laboratories, Detroit, MI). These  
10      animals were boosted (SQI) 4 weeks later with 25 µg antigen (Ag; lot 1) prepared in similar fashion using Incomplete Freund's Adjuvant (ICFA; Difco Laboratories, Detroit, MI). Mice were bled via the tail 9 days later and serum antibody (Ab) titres determined by enzyme-linked immunosorbent assay (EIA). As the 1/2 maximal titre for each mouse rose above 5000, individual animals  
15      were selected for the hybridoma preparation. The three animals (#7, 8 and 9) which were used to generate the hybrids of interest (#71A and 73A) required additional  
20      boosts at 5 weeks and again at 29 weeks using 12.5 µg Ag (lot 1) and 25 µg Ag (lot 2) respectively. These boosts were performed in the same manner as the initial boost; that is, as an emulsion in 50% vol/vol ICFA. Serum Ab titres continued to be monitored 9 days following each  
25      boost. The final titres of these mice prior to fusion were 5026, 6842, and 12,945 for animals 7, 8 and 9, respectively.

C. Cell Fusion

30       Animals 7, 8 and 9 were intravenously injected with 25 µg of sHuEPOR (lot #3) 8 weeks following the final boost. Four days later, mice were sacrificed by carbon dioxide and spleens collected under sterile conditions into 25 ml Dulbecco's Modified Eagle's Medium containing  
35      200 U/ml Penicillin G, 200 µg/ml Streptomycin sulfate, and 4 mM glutamine (2x P/S/G DMEM). The spleens were

- 23 -

trimmed of excess fatty tissue, then rinsed through 3 dishes of clean 2x P/S/G DMEM. They were next transferred to a sterile stomach bag (Tekmar, Cincinnati, OH) containing 10 ml of 2x P/S/G DMEM, and 5 disrupted to single cell suspension with the Stomacher Lab Blender 80 (Seward Laboratory UAC House; London, England). As cells were released from the spleen capsule into the media, they were removed from the bag and passed through a 70  $\mu$ m nylon mesh cell strainer (Becton Dickinson and Company; Lincoln Park, NJ). Fresh media was replaced in the bag and the process continued until the entire cell content of the spleens were released. These splenocytes were washed 3 times by centrifugation at 225 x g for 10 minutes. In the first 10 fusion, splenocytes from animal #9 were used; in the second fusion, splenocytes from animals #7 and 8 were 15 pooled.

Concurrently, log phase cultures of Sp2/0-Ag14 mouse myeloma cells (available from the American Type 20 Culture Collection, Rockville, MD under accession no. CRL 1581) grown in complete medium (DMEM, 10% fetal bovine serum, 2 mM glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 10 mM Hepes Buffer; Gibco Laboratories, Inc., Grand Island, NY), were washed 25 in similar fashion. From this myeloma population, 4 x  $10^7$  cells (fusion 1) or 8 x  $10^7$  cells (fusion 2) were taken, mixed with the suspension of splenocytes, and pelleted once again. The media was aspirated from the cell pellet and 2 ml of polyethylene glycol (PEG 1500 30 Mwt; Boehringer Mannheim Biochemicals, Indianapolis, IN) for fusion 1 or 3.5 ml of PEG for fusion 2 at 37°C were gently mixed into the media over the course of 1 minute. Thereafter, an equal volume of 2x P/S/G DMEM was slowly 35 added. The cells were allowed to rest at 37°C for 2 minutes, then an additional 9 ml of 2xP/S/G DMEM added. The cells were again set at 37°C for 4 minutes.

- 24 -

Finally, 30 ml of 2x P/S/G DMEM was added to the cell suspension, and the cells pelleted by centrifugation. Media was aspirated from the pellet and the cells gently resuspended into approximately 56 ml (fusion 1) or 74 ml (fusion 2) of complete medium containing 100 U/ml Penicillin G and 100 µg/ml Streptomycin Sulfate. Cells were distributed over 10 96-well flat bottom tissue culture plates (Becton Dickinson Labware; Lincoln Park, NJ) by single drops from a 5 ml pipette. Plates were 5 incubated in humidified conditions at 37°C, 5% CO<sub>2</sub>, overnight. The next day, an equal volume of selection medium was added to each well. Selection consisted of 10 0.1 mM hypoxanthine, 4 x 10<sup>-4</sup> mM aminopterin, and 1.6 x 10<sup>-2</sup> mM thymidine in complete medium. The fusion plates 15 were incubated for 7 to 10 days with 2 changes of medium during this time; HAT selection medium was used after each fluid change. Tissue culture supernatants were taken from each hybrid-containing well and tested by EIA 20 for specific antibody reactivity to sHuEPOR. 96 wells which were positive in EIA were subjected to further screening.

#### D. Dot Blots

Dot blots of reduced sHuEPOR (lot #4) were used as 25 a secondary screening method for EIA positive hybridomas. The Dot Blot SF Microtitration Apparatus (Bio-Rad Laboratories, Inc.; Richmond, CA) was set-up according to the instruction manual; nitrocellulose membranes (9 x 12 cm; Bio-Rad Laboratories, Inc.; 30 Richmond, CA) were employed. Antigen was first prepared by boiling for 5 minutes under reducing conditions with 2-mercaptoethanol (5% vol/vol; Bio-Rad Laboratories, Inc.; Richmond, CA) in Tris-buffered saline solution (TBS; 10 mM Tris pH 7.5, 154 mM NaCl, 0.01% wt/vol Na 35 azide). Twenty-five ng of sHuEPOR (lot #4) was loaded into each well and aspirated through the nitrocellulose

- 25 -

membrane for binding. The wells were filled with 250  $\mu$ l Blotto-Tween solution (block solution; 2% wt/vol non-fat dry milk, 50 mM Tris, pH 7.5, 25 mM NaCl, 0.1 mM EDTA, 0.09% vol/vol Tween 20, 0.01% vol/vol anti-foam A) and 5 incubated at RT for 30 minutes. Block solution was aspirated from the wells and the procedure repeated for a second time to ensure complete blocking of non-specific sites on the membrane. This was followed by 3 washes through the membrane with D-PBS containing 0.1% 10 vol/vol polyoxyethylene sorbitan monolaurate (Tween-20; Bio-Rad Laboratories, Inc.; Richmond, CA). Ninety-five  $\mu$ l of EIA-positive hybridoma conditioned medium was next added to each well and incubated for 45 minutes at RT. Wells were washed 3x with TBS-Tween (20 mM Tris, pH 7.5, 15 50 mM NaCl, 0.02% vol/vol Tween 20) and 2x with TBS-Tween (20 mM Tris, pH 7.5 0.5 M NaCl, 0.09% vol/vol Tween 20) at 250  $\mu$ l per wash, aspirating through the membrane after each addition. One-hundred  $\mu$ l of goat 20 anti-mouse IgG, heavy- and light-chain specific, 25 HRP-conjugated secondary antibody (1:1000 diluted in TBS-Tween; Boehringer Mannheim Biochemicals; Indianapolis, IN) was incubated in each well for 45 min at RT. Membranes were washed as before, removed from the blot apparatus, dipped into prepared Enhanced Chemiluminescent Reagent (ECL reagent; Amersham Life Sciences, Corporation; Arlington Heights, IL), and exposed to X-OMAT AR film (Kodak Scientific Imaging, Rochester, New York). Fifteen seconds later, the film 30 was removed from film cassettes and developed. Each well was scored 3+ to 0 based on intensity of dots for individual hybridoma supernatants.

- 26 -

EXAMPLE 4  
Anti-EPOR antibody binding to EPOR

5 5. A. Antibody binding to EPO-R by BIACore Analysis

Real-time biospecific interaction analysis (BIA, Pharmacia Biosensor AB, Uppsala, Sweden) based on surface plasmon resonance (SPR) (Fiagerstam et al. J. Mol. Recognition 3, 208 (1990); Malmborg et al. Scand. J. Immunol. 35, 643 (1992)) was used to screen the ELISA positive monoclonal antibodies.

Soluble HuEPOR prepared as described in Examples 1 and 2 was covalently coupled to the sensor chip CM5 via the primary amine group. The immobilization was 15 performed at a flow of 5 ul/min in HBS (10mM HEPES pH7.4, 150 mM NaCl, 3.4 mM EDTA, 0.05% BIACore surfactant P-20). The carboxylated matrix of the sensor chip was first activated with a 40 ul injection of 1:1 mixture of EDC (400 mM N-ethyl-N-(dimethylamine-20 propyl)carbodiimide in water, Pharmacia Biosensor AB) and NHS (100mM N-hydroxysuccinimide in water, Pharmacia Biosensor AB). 65 ul of soluble EPO-R(50 ug/ml in 10mM Na-acetate pH4.0) was injected to immobilize onto the sensor chip. The excess reactive groups of the sensor 25 chip were deactivated with an injection of 50 ul of ethanolamine (Pharmacia Biosensor AB)

Each analysis cycle included an injection of 20ul of hybridoma supernatant, followed by injection of 10ul of 10mM HCl for regeneration of the chip. The SPR 30 response is measured in Resonance Units (RU). For most proteins, 1000 RU corresponds to a surface concentration of approximately 1 ng/mm<sup>2</sup>. Results of screening 96 wells which were positive in EIAs are shown in Table 1. In these experiments, background is typically about 20 35 RU. Binding to EPOR is significant at 50 RU and above.

- 27 -

**TABLE 1**  
**EPO-R Monoclonal Antibodies**

ANTIBODY (1)	BIACORE (2)	BIACORE (3) COMPETITION GROUP	FACS (4) MEAN FLOURE- SCENCE	Inhibition of EPO Activity (5)	Stimulation of UT7-EPO Cells (6)
1	98	A	-	-	-
2	8	NT	-	NT	NT
3	7	NT	-	NT	NT
4	65	NT	-	NT	NT
5	13	NT	-	NT	NT
6	9	NT	-	-	-
7	89	C	-	NT	NT
8	46	NT	-	NT	NT
9	29	NT	-	NT	NT
10	69	NT	-	NT	NT
11	4	NT	-	NT	NT
12	153	C	-	NT	NT
13	1499	B	-	NT	NT
14	87	NT	-	NT	NT
15	29	NT	-	NT	NT
16	8	NT	-	NT	NT
17	7	NT	-	NT	NT
18	46	NT	-	-	-
19	9	NT	-	NT	NT
20	7	NT	-	NT	NT
21	49	NT	-	NT	NT
22	8	NT	-	NT	NT
23	4	NT	-	-	-
24	26	NT	-	NT	NT
25	8	NT	-	NT	NT
26	84	NT	-	NT	NT
27	2	NT	-	NT	NT
28	11	NT	-	NT	NT
29	1	NT	-	NT	NT
30	270	A	-	-	-
31	16	NT	-	-	NT
32	18	NT	-	NT	NT
33	15	NT	-	NT	NT
34	25	NT	-	NT	NT
35	363	A	-	NT	NT
36	4	NT	-	NT	NT
37	16	NT	-	-	-
38	13	NT	-	NT	NT
39	574	B	-	-	-
40	15	NT	-	NT	NT
41	22	NT	-	NT	NT
42	23	NT	-	NT	NT
43	6	NT	-	NT	NT
44	13	NT	-	NT	NT
45	13	NT	-	NT	NT

- 28 -

TABLE 1 (Con't)

ANTIBODY (1)	BIACORE (2)	BIACORE (3) COMPETITION GROUP	FACS (4) MEAN FLOURE- SCENCE	Inhibition of EPO Activity (5)	Stimulation of UT7-EPO Cells (6)
46	7	NT	-	NT	NT
47	10	NT	-	NT	NT
48	5	NT	-	NT	NT
49	69	NT	-	NT	NT
50	345	C	-	-	-
51	31	NT	-	NT	NT
52	6	NT	-	NT	NT
53	130	A	-	NT	NT
54	13	NT	-	NT	NT
55	34	NT	-	NT	NT
56	11	NT	-	NT	NT
57	10	NT	-	NT	NT
58	15	NT	14.99	+	?
59	10	NT	-	NT	NT
60	10	NT	-	NT	NT
61	48	NT	-	NT	NT
62	814	A	-	-	-
63	1539	B	-	NT	NT
64	1222	C	-	NT	NT
65	-5	NT	-	+/-	?
66	975	C	-	NT	NT
67	1000	A	-	-	?
68	495	C	-	NT	NT
69	877	A	-	-	-
70	789	A	-	-	?
71	1584	C	23.55	+ (7)	+++
72	1190	B	-	-	-
73	354	C	13.71	-	+
74	408	A	18.53	-	-
75	947	B	-	NT	NT
76	6	NT	-	NT	NT
77	434	C	-	-	-
78	119	A	-	NT	NT
79	8	NT	-	NT	NT
80	11	NT	-	NT	NT
81	-4	NT	-	NT	NT
82	4	NT	-	NT	NT
82B	-13	NT	NT	NT	NT
83	1025	C	-	-	-
84	5	NT	-	NT	NT
85	11	NT	-	NT	NT
86	859	C	-	NT	NT
87	4	NT	12.81	-	-
88	4	NT	-	+/-	-
89	-1	NT	-	+/-	-
90	4	NT	-	NT	NT
91	0	NT	-	-	-

- 29 -

TABLE 1 (Con't)

ANTIBODY (1)	BIACORE (2)	BIACORE (3) COMPETITION GROUP	FACS (4) MEAN FLOURE- SCENCE	Inhibition of EPO Activity (5)	Stimulation of UT7-EPO Cells (6)
92	-3	NT	-	NT	NT
93	2	NT	-	NT	NT
94	5	NT	-	NT	NT
95	417	A	-	NT	NT
96	7	NT	-	NT	NT

5 Tissue culture medium conditioned by hybridomas secreting the indicated antibodies were tested with the assays indicated. Supernatants containing all the antibodies shown gave a positive signal in ELISA assays. +++, ++, + indicate a positive response with +++; indicating those having the greatest effect.

10 - indicates a response less than or equal to the response of control medium. NT indicates samples were not tested. ? indicates samples that could not be assigned a response.

15 (1) Antibodies 1-61 are from mice number 7 and 8. Antibodies 62-96 are from mice number 9.

(2) Response units by Mabs using biacore chip with attached sHuEPOR.

(3) Competition on BIACORE was to anti sHuEPOR Mab 1G2.

20 sHuEPOR bound to a sensor chip was incubated with 1G2 then effect on Mab binding compared to binding to EPOR not preincubated with 1G2 was determined. Antibodies whose binding was completely blocked (80-100%) are A. Antibodies whose binding was blocked 50-80% are C. Antibodies whose binding was blocked less than 50% are B.

25 (4) Values for antibodies that gave cells a mean fluorescence greater than the control (12.73) are shown -- indicates antibodies with a mean fluorescence less than or equal to control.

(5) Inhibition of  $^3$ H uptake by UT7-EPO cells. 30 munits of EPO and varying amounts of antibody were incubated with cells. After an overnight incubation cells were pulse labeled with  $^3$ H thymidine and the amount of counts taken up were determined. A positive response was defined as one that had a progressive decrease with increasing amounts of antibody

- 30 -

(6) Stimulation of  $^3\text{H}$  uptake by UT7-EPO cells. Varying amounts of antibody were incubated with cells. After an overnight incubation, cells were pulse labeled with  $^3\text{H}$  thymidine and the amount of counts taken up were determined. A positive response was defined as one that had a progressive increase in incorporation with increasing amounts of antibody.

(7) Inhibition was at concentrations higher than required to activate.

10

#### B. Epitope Competition Analysis

The sensor chip which was immobilized with sHuEPOR could be saturated by an injection of 65  $\mu\text{l}$  of hybridoma supernatant 1G2. 1G2 is a monoclonal antibody raised to sHuEPOR using procedures described in Example 3. Each analysis cycle included injections of 20  $\mu\text{l}$  of the hybridoma supernatant with and without one epitope being saturated by the injection of 65  $\mu\text{l}$  of 1G2. The ratio of the binding signal in RU of 20  $\mu\text{l}$  injection after 1G2 saturation versus the binding signal in RU of 20  $\mu\text{l}$  injection alone is defined as % blocking by 1G2. Those antibodies with 80-100% blocking are assigned as group A, those with less than 50 % blocking as group B, and those with 50-80% blocking as group C. The results are shown in Table 1.

#### C. Antibody Binding to d40EPOR on Transfected CHO Cells by Fluorescence-activated Cell Sorting (FACS) Analysis

30 Hybridoma supernatants raised against EPOR were tested for binding to EPO receptor on the surface of pDSR $\alpha$ EPORd40 transfected CHO cells by FACS analysis. CHO cells transfected with DNA encoding d40 EPO receptor were constructed as described in Example 1. CHO/EPOR

35 cells were scraped from tissue culture dishes and resuspended as single cells in a solution of PBS/0.5% BSA and were then distributed into a 96 well round-bottom plate at approximately  $3 \times 10^5$ /well. The plate

- 31 -

was then placed in the centrifuge at 1000 x g for 5 min. After centrifugation, the PBS/BSA supernatant was removed and each of the pelleted cells were resuspended in either a control media or in one of the EPOR 5 hybridoma supernatants. The cells were incubated at 4° C for 1 hour. After the incubation, cells were washed with PBS/BSA and then resuspended in a solution of fluoresceine isothiocyanate (FITC) labelled Goat anti Mouse monoclonal antibody (Southern Biotech, Birmingham 10 Ala.). The cells were incubated again at 4° C for 1 hour, washed and analyzed by FACS. Of the 96 supernatants tested, five had a mean cell fluorescence greater than control media (see Table 1). Mab 71 gave the highest level of fluorescence followed by Mabs 74, 15 58, 73 and 87. No other supernatants tested exhibited fluorescence above control values.

#### EXAMPLE 5

#### Purification of anti-EPOR antibodies and Fab fragments

##### A. Ascites Production

Balb/c mice (Charles Rivers Laboratories, 25 Wilmington, MA), greater than 5 weeks of age were primed with 2,4,10,14-tetramethyl-pentadecane (Pristane; Sigma, St. Louis, MO) 7 to 10 days prior to injection of cell lines. Each mouse received a single intraperitoneal injection of 0.5 ml; 10 to 20 animals were injected for 30 each cell line for which ascites fluid was to be prepared.

Hybridoma lines grown in complete medium until confluence was attained, were washed once with D-PBS then counted using a Neubauer Hemacytometer. Each mouse 35 was then intraperitoneally injected with  $10^7$  cells, and maintained on Rodent Lab Chow and water ad libitum

- 32 -

until ascites fluid developed. Mice were monitored for maximum ascites formation, sacrificed under CO<sub>2</sub>, and tapped for fluid collection using an 18G needle inserted into the fluid-filled cavity. The fluid was clarified 5 by centrifugation at 225 x g for 15 min or for 3 minutes in a microcentrifuge (Eppendorf). Four ml aliquots were then stored at -20°C until purified by Protein-A column chromatography.

10 B. Protein-A Purification of Monoclonal Antibodies:

10 Immunoglobulin from 4 ml of ascites fluid or 10 ml of hybridoma conditioned medium was purified by Protein-A column chromatography. The Bio-Rad Monoclonal Antibody Purification System II (MAPS II; Bio-Rad 15 Laboratories; Richmond, CA) was used. Briefly, 5 ml of Affi-gel Protein-A suspension was settled into a 1 x 10 cm disposable glass column. The Protein-A gel was washed with approximately 30 ml of D-PBS then prepared by running 20 ml of Binding Buffer (MAPS II Binding 20 Buffer; Bio-Rad) through the column. Ascites fluid or conditioned medium diluted 1:1 with binding buffer was then added to the top of the column and allowed to flow through. After binding of immunoglobulin to Protein-A, the unbound fraction was discarded. The column was next 25 rinsed of unbound protein with 30 ml of binding buffer to yield an absorbance at 280 nm of less than 0.01. The immunoglobulin-containing fraction was then eluted with Bio-Rad Elution buffer, approximately 30 ml. This fraction was buffer-exchanged overnight at 4°C by 30 dialysis against 4 liters D-PBS. The resulting PBS-equilibrated immunoglobulin was concentrated by centrifugation at 1700 x g in Centricon Concentrator units (Amicon Inc., Beverly, MA).

- 33 -

C. Fractionation of the Antibody-binding Domain

Protein-A purified immunoglobulin was further fractionated into its 2 component parts, the crystallizable fraction (Fc) and the antibody-binding fraction (Fab), using a Pierce ImmunoPure Fab Preparation kit (Pierce Chemical Company, Rockford, IL). The protein-A purified immunoglobulin was dialyzed into 20 mM phosphate/10 mM EDTA buffer at pH 7.0, then concentrated to approximately 20 mg/ml. Ten mg of immunoglobulin was fractionated. Immobilized papain gel was rinsed twice with digestion buffer containing 42 mg cysteine in 12 ml phosphate buffer as supplied. The immunoglobulin sample was then added to the gel and incubated at 37°C, on a rotating shaker, overnight. The solublized Fab was separated from the Fc and undigested immunoglobulin by protein-A purification; unbound fraction was collected here as the Fab sample. This unbound portion was dialyzed overnight against 4 liters D-PBS at 4°C, and concentrated as before.

20

EXAMPLE 6

Mapping of Mab 71 epitope on EPOR

25 Overlapping synthetic peptides 17 to 30 amino acids in length were made that spanned residues 1 to 224 of the human EPO receptor, where residue 1 is proline and residue 224 is aspartic acid. The ten different peptides overlapped by six amino acids at both ends.

30 The sequences of the peptides and their location within the human EPO-R amino acid sequence are as follows:

SE-1 PPPNLPDPKFESKAALLAARGPEELCFTE  
(residues 1-30)

35 SE-2A LLCFTERLEDLVCFWEEA  
(residues 25-42)

- 34 -

SE-2B CFWEEAASAGVGPGNYSF  
(residues 37-54)  
SE-3 PGNYSFSYQLEDEPWKLCLRLHQAPTARGAV  
(residues 49-78)  
5 SE-4 TARGAVRFWCSLPTADTSSFVPLELRVTAA  
(residues 73-102)  
SE-5 LRVTAASGAPRYHRVIHINEVVLLDAPVGL  
(residues 97-126)  
SE-6 DAPVGLVARLADES GHVVLRVLPPPETPMT  
10 (residues 121-150)  
SE-7 PETPMTSHIRYEVDVSAGNGAGSVQRVEIL  
(residues 145-174)  
SE-8 QRVEILEGRTECVLSNLRGRTRYTFAVRAR  
(residues 169-198)  
15 SE-9 FAVRARMEAPSFGGFWSAWSEPVSLLTPSDL  
(residues 193-224)

Polystyrene wells (Costar, Cambridge, MA) were  
coated with the above EPO-R peptides at concentrations  
20 of 100 $\mu$ g/ml, 20  $\mu$ g/ml and 0.8  $\mu$ g/ml respectively in  
carbonoate-biocarbonate buffer (0.015M Na<sub>2</sub>CO<sub>3</sub>, 0.035M  
NaHCO<sub>3</sub>, pH 9.2). The plate was incubated at room  
temperature (RT) for 2 hours then overnight at 4°C.  
Soluble HuEPOR was coated at concentrations of 10 $\mu$ g/ml,  
25 2 $\mu$ g/ml, 0.4 $\mu$ g/ml and 0.08 $\mu$ g/ml as positive controls  
under the same conditions. After blocking with 5% BSA  
in PBS at RT for 30 minutes, the plate was incubated  
with Mab 71 purified as described in Example 5 at a  
concentration of 5 $\mu$ g/ml in 1% BSA at RT for 2 hours.  
30 After washing with washing buffer (Kirkegaard and Perry  
Labs, Inc.) the plate was incubated with 1:1000 dilution  
of Goat anti-mouse IgG conjugated with horse Radish  
peroxidase (Boehringer Mannheim) for one hour at RT.  
The plate was washed and developed with ABTS (Kirkegaard  
35 and Perry Labs, Inc.) substrate solution. Colorimetry  
was conducted at 405 nm. The results of Mab binding to

- 35 -

the synthetic peptides are shown in Figure 1 and indicate that Mab 71 binds significant amounts of peptide SE-3 (amino acid residues 49 to 78 inclusive of human EPO-R) compared to the other peptides tested.

5 This indicates that Mab 71 binds to a region of the  
human EPO-R containing or overlapping residues 49 to 78.

**EXAMPLE 7**

## 10 Activity of anti-EPOR antibodies in cell proliferation assays

Antibodies in conditioned medium prepared as described above were assayed for their ability to stimulate uptake of  $^3\text{H}$ -thymidine by UT7-EPO cells (Komatsu et al., *supra*). UT7-EPO cells are responsive to EPO and express human EPO receptors on their cell surface. UT7-EPO cells were grown in Growth medium (1X Iscove's Modified Dulbecco's Medium with L-glutamine, 25 mM HEPES buffer, and 3024 mg/L sodium bicarbonate, but without either alpha-thioglycerol or beta-mercaptoethanol (GIBCO) / 10% v/v Fetal Bovine Serum / 1% v/v L-glutamine-Penicillin-Streptomycin solution (Irvine Scientific) / 1 Unit/ml rHuEPO ) to approximately  $3 \times 10^5$  cells/ml. Cells were collected by centrifugation (approx. 500xG) washed twice with phosphate buffered saline and resuspended at  $5 \times 10^4$  cells/ml in Assay medium (1x RPMI Medium 1640 without L-glutamine (Gibco) / 1% L-glutamine / 4% fetal bovine serum). Test samples or EPO standard (rHuEPO), 100  $\mu\text{L}$  diluted in assay medium at least 5-fold, were added to wells in a 96 well microtiter plate. 50  $\mu\text{L}$  cells were then added (5000 cells/well) and plates were incubated in a humidified incubator at 37°C and 5% CO<sub>2</sub>. After 72 hours, 50  $\mu\text{L}$  methyl- $^3\text{H}$ -Thymidine (1 mCi/ml; 20 Ci/mMole) diluted 1:100 in assay medium was added. Cells were incubated

- 36 -

for an additional 4 hours at 37°C and 5% CO<sub>2</sub>. Labeled cells were harvested onto glass fiber filtermats using a PHD cell harvester(Cambridge Technology Inc.) and deionized water as a washing solution. Filters were 5 rinsed a final time with 2-propanol then dried and counted in a Beckman Model LS6000IC scintillation counter.

Conditioned medium from tissue culture plates containing antiEPOR Mabs were tested for their ability 10 to stimulate proliferation as described above. Samples at several dilutions were tested. Positive responses were defined as those that stimulated thymidine uptake at least 2-fold over background levels and also resulted in decreasing stimulation as the samples were diluted. 15 As shown in Table 1, two samples out of 24 tested gave a positive response (Mabs 71 and 73). Four samples may have a weak stimulatory activity (?) in Table 1). The remaining samples did not give a significant increase over background. A polyclonal serum from the mouse used 20 to generate monoclonals also stimulated thymidine uptake. This suggests that the polyclonal antibody in this serum was also capable of stimulating proliferation of UT7-EPO cells.

The supernatants were also tested for their ability 25 to inhibit EPO-induced stimulation of thymidine uptake by UT7-EPO cells. Cells were incubated with 25 munits/ml rHuEPO and varying amounts of antibody containing conditioned medium. Thymidine uptake was measured as described above. The results are shown in 30 Table 1. Most antibodies did not significantly differ from control medium. Of the antibodies showing inhibition of thymidine uptake, two samples (Mabs 58 and 73) showed definite inhibition while three samples (Mabs 65, 88 and 89) showed possible inhibition. Mab 73 35 inhibited at the highest doses but at lower doses it stimulated thymidine uptake over control values.

- 37 -

EXAMPLE 8

5 Activation of EPOR by  
anti-EPOR antibodies and fragments

A. UT7-EPO Proliferation Assay

Mabs 71 and 73 were purified as described in Example 5. Proliferative activity was determined with 10 UT7-EPO thymidine uptake assays described in Example 7. Both Mabs 71 and 73 stimulated uptake by UT7-EPO cells in a dose dependent manner as did rHuEPO (see Figure 2). Activity was reduced at high doses of Mab 71. Peaks in stimulatory activity were observed at doses of 1-2  $\mu$ g/ml for Mab 71 and >100 $\mu$ g/ml for Mab 73. A nonneutralizing control antibody (AntiEPO Mab F12) did not stimulate which suggests that the stimulation is specific for EPO receptor antibodies.

20 B. EPO cold displacement assays.

Antibodies to the EPO receptor may bind to the same region as EPO binds. To test this possibility, cold displacement assays were performed using OCIM1 cells. OCIM1 cells are from human origin and known to contain 25 EPO receptors on their cell surface (Broudy et al. Proc. Nat. Acad. Sci. USA 85, 6517 (1988)). Cells were grown in OCIM1 growth medium (Iscove's modified Dulbecco medium(IMDM)/10% fetal bovine serum/1% pen-strep-fungisone) to approximately 2-5x10<sup>5</sup> cells/ml. Cells 30 were collected by centrifugation, washed two times in binding buffer (RPMI 1640/ 1%BSA/ 25mM HEPES pH 7.3) then resuspended in binding buffer containing 0.1% azide and 10  $\mu$ g/ml cytochalisin B at 1-2x10<sup>7</sup> cells/ml. Cells (100 $\mu$ L) in 96 well tissue culture plates were then 35 incubated with 10 $\mu$ L sample and 10 $\mu$ L <sup>125</sup>I-EPO (Amersham high specific activity; 3000Ci/mMole, 2  $\mu$ Ci/ml) in a 37°

- 38 -

humidified tissue culture incubator. After 3 hours cells were centrifuged through phthalate oil (60:40 (v/v) dibutyl/dinonyl phthalate) in titer tubes. The tubes containing cells were quick frozen in a dry ice-ethanol 5 bath and the cell pellet was clipped and then counted in a LKB 1277 gammamaster automatic gamma counter.

Figure 3 shows the results of the cold displacement experiment. Increasing amounts of  $^{125}\text{I}$ -EPO were displaced from EPO receptors on cells as the amount of 10 added unlabeled rHuEPO was increased. In a similar manner, Mab 71 purified as described in Example 5 also displaced increasing amounts of  $^{125}\text{I}$ -EPO with increasing amounts of antibody. In this case, approximately 4,000 fold more Mab 71 was needed than rHuEPO to displace 15 equivalent amounts of  $^{125}\text{I}$ -EPO. In contrast Mab 73 showed indications of displacement at the highest doses but a nonneutralizing anti rHuEPO Mab (F12) did not significantly displace. These results indicate that Mab F12 did not interfere with binding of EPO to its 20 receptor but Mab 71 and 73 do. This result also indicates that Mab 71 binds to the EPO receptor and activates it by binding at or close to the EPO binding site.

25 C. Comparison of activities of Mab 71 and Fab 71

EPO receptor fragments of Mab 71 were prepared as described in Example 5. The preparations were characterized by SDS gel electrophoresis (Laemmli et al. 30 *Nature* 227, 680 (1970) as shown in figure 4. Samples were boiled in 2% SDS containing sample buffer with or without 0.7M 2-mercaptoethanol, to distinguish reduced (2-mercaptoethanol) from nonreduced (no 35 2-mercaptoethanol) proteins, then run on 12.5% acrylamide SDS gels. The gels were stained with coomassie blue to visualize the proteins. The sizes of the proteins were estimated by comparing their

- 39 -

mobilities to the mobilities of protein standards. Mabs 71 and 73 separated into light and heavy chains when run under reducing conditions. The heavy chains were approximately 52 KDa. The light chain for 73 was 5 slightly smaller (28 KDa) than for Mab 71 (28.5 KDa). The Fab fragments also had two chains: 28.3 and 27.3 KDa for Fab 71 and 27.5 and 26.5 KDa for Fab 73. When these Fab fragments were run under non reducing conditions, the sizes of Fabs 71 and 73 were approximately 48 and 47 10 KDa respectively. This indicates that the Fab fragments are monovalent, the complex has one each of the light and heavy chains. In contrast the mobilities on nonreducing SDS gels for Mabs 71 and 73 indicated that their sizes were approximately 200 KDa. This indicates 15 that these Mabs are bivalent, there are two each of the heavy and light chains.

To see if monovalent Fab 71 fragments would activate the EPO receptor, Mab 71 and the Fab 71 fragment were incubated with UT7-EPO cells and thymidine 20 uptake was measured as described in Example 7. As shown in figure 5, both rHuEPO and Mab 71 stimulated thymidine uptake. However the monovalent Fab 71 fragment did not. A control monoclonal antibody raised against an unrelated receptor (Her2/neu) also did not stimulate 25 thymidine uptake. This indicates that the antibodies must be bivalent in order to activate the receptor.

D. Stimulation of thymidine uptake by Mab 71 and Fab 71 in the presence of rHuEPO.

30 The fact that Mab 71 inhibits binding of EPO to EPO receptors suggested that the antibody may not activate the EPO receptor in the presence of EPO. To test this possibility UT7-EPO cells were incubated with 30 munits/ml rHuEPO and varying amounts of purified Mab 71, 35 Fab 71 or Mab control (raised against Her2/neu). Thymidine uptake was measured as described above. As

- 40 -

shown in figure 6 Both Mab 71 and Fab 71 inhibited thymidine uptake at high doses. However at doses between approximately 30 and 3000  $\mu$ g/ml, Mab 71 stimulated thymidine uptake above levels stimulated by 5 rHuEPO alone. Fab 71 and control antibodies did not have this effect. This indicates that Mab 71 and rHuEPO can have an additive effect in EPO receptor activation.

10

#### EXAMPLE 9

##### Stimulation of erythroid colony formation by anti-EPOR antibodies

To see if purified Mab 71 would stimulate formation 15 of erythroid cells from precursors in peripheral blood a BFUe assay was done. To purify erythroid cell precursors, normal human donors were lymphopheresed according to standard protocol. The lymphopheresed cells (250 ml) were washed with 250 ml Hank's Balanced Salt 20 Solution (HBSS). The cells were resuspended in HBSS and separated by density centrifugation over a gradient(Ficoll-paque) for 30 min at 500xg. The low density cells(LD) were collected from the gradient and washed with 500ml HBSS and resuspended in PBS 25 supplemented with 0.5% bovine serum albumin and 5mM EDTA at a concentration of  $5 \times 10^8$  cells/ml. The LD cells were then further purified using a CD34 progenitor Cell Isolation Kit (QBend/10) made by Miltenyi Biotech GmbH. In brief cells were tagged with an anti CD34 monoclonal 30 antibody they were then bound to magnetic microspheres according to protocol. The tagged cells were next passed through pre-filled MiniMacs separation columns, the columns were washed and the CD34+ cells were then eluted from the column. This process was repeated once more to 35 achieve a higher purity of CD34+ cells. The *in vitro* assay was done on the purified CD34+ cells as described

- 41 -

by Iscove et. al. (J. Cell. Physiol 83, 309 (1974)) with the following modifications. The culture medium was obtained from Gibco BRL (Human bone marrow stem cell proliferation kit; Grand Island, NY). To plate out 5 duplicate 1ml samples on 35x100mm tissue culture plates, an excess of 3 ml was prepared in 17x100 sterile polystyrene tubes. Each tube received 2.5 ml Stem Cell Growth medium, 0.1 ml CD34<sup>+</sup> cells (resuspended at 90,000 cells/ml) 0.015 ml Stem Cell Factor (20 µg/ml), and a 10 combination of sample and Stem Cell Dilution medium equaling 0.385 ml. The tubes were vortexed and allowed to settle to allow bubbles to rise. The contents were then aliquoted using a 3ml syringe with a 17x1-1/2 needle. The plates were incubated at 37°C and 10% CO<sub>2</sub> 15 in a humidified tissue culture incubator. Erythroid colonies (orange to red in color) were scored after 21 days. No erythroid colonies were seen in plates lacking EPO or Mab 71. rHuEPO (30mUnits/plate) gave an excess of 400 colonies per plate. Mab 71 also produced 20 erythroid colonies. Peak activity was seen at 2-6 µg/ml. This result indicates that Mab 71 stimulates formation of erythroid colonies.

The activity of purified Mab 71 was also tested for the ability to form erythroid colonies using serum free 25 growth conditions in methylcellulose. CD34<sup>+</sup> cells were isolated as described above and incubated using the serum free growth medium described in co-pending and co-owned U.S. Serial No. 08/079,719, hereby incorporated by reference, with the following modifications. The assay 30 tubes were set up without using extracellular matrix molecules, hydrocortisone, and the growth factors EGF, FGF, and PDGF. As described above 3 mL of sample was prepared to plate out duplicate 1 mL samples on plates. Each tube received .030ml each of 100x Stock Solutions 35 (2-Mercaptoethanol, nucleosides, cholesterol, Sodium-Pyruvate, Hu-Transferrin, lipids, Hu-Insulin), 0.4ml

- 42 -

deionized BSA (15%), 0.015ml SCF (20 ug/ml), 0.1 ml CD34+ cells (resuspended at 300,000 cells/ml), 1.080ml methylcellulose (2.3%), and a combination of sample and IMDM equaling 1.195ml where the sample did not exceed 5 150  $\mu$ l. The plates were then incubated as described above and colonies were scored after 21 days. Erythroid colonies were observed when grown in the presence of EPO or Mab 71 but not under conditions lacking these two factors. An example of the erythroid colony types seen 10 is shown in figure 7. Colonies incubated with 25 munits of rHuEPO looked similar to those grown with 2.1  $\mu$ g/ ml of purified Mab 71. Higher doses of rHuEPO gave larger colonies. A dose response curve is shown in Figure 8. Mab 71 had a peak in activity at doses between 1 and 5 15  $\mu$ g/ml. Lower and higher doses resulted in fewer erythroid colonies. A control monoclonal antibody raised to Her2/Neu did not produce any colonies over this dose range. This result indicates that the Mab 71 will stimulate the formation of erythroid colonies from 20 erythroid precursors and that there is not an additional requirement for serum. Thus Mab 71 can stimulate differentiation of erythroid precursors into erythroid cells.

25

\* \* \*

While the present invention has been described in terms of the preferred embodiments, it is understood that variations and modifications will occur to those 30 skilled in the art. Therefore, it is intended that the appended claims cover all such equivalent variations which come within the scope of the invention as claimed.

- 43 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Amgen Inc.

(ii) TITLE OF INVENTION: ANTIBODIES WHICH ACTIVATE AN  
ERYTHROPOIETIN RECEPTOR

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Amgen Inc.  
(B) STREET: 1840 Dehavilland Drive  
(C) CITY: Thousand Oaks  
(D) STATE: California  
(E) COUNTRY: USA  
(F) ZIP: 91320-1789

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Winter, Robert B.  
(C) REFERENCE/DOCKET NUMBER: A-307

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 42 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

- 44 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTCCAAGCTT GCCGTCACCA TGGACCACCT CGGGGCGTCC CT

42

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CAGGTCTAGA TTACTAGGGA TCCAGGTCGC TAGGC

35

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AGGTCGACTA CTAGTAGTCA GTTGAGA

27

- 45 -

WHAT IS CLAIMED IS:

1. An antibody or fragment thereof which activates an erythropoietin receptor.
- 5 2. The antibody of Claim 1 wherein the erythropoietin receptor is a mammalian erythropoietin receptor.
- 10 3. The antibody of Claim 1 wherein the erythropoietin receptor is a human erythropoietin receptor.
4. The antibody of Claim 1 which is a monoclonal antibody.
- 15 5. The antibody of Claim 1 which is a humanized antibody.
6. The antibody of Claim 1 which is a human antibody.
- 20 7. The antibody of Claim 1 having a detectable label.
8. A hybridoma cell line capable of producing the monoclonal antibody of Claim 4.
- 25 9. An antibody or fragment thereof which recognizes an epitope on an erythropoietin receptor which is recognized by the monoclonal antibody produced by the hybridoma cell line ATCC No. HB11689 or ATCC No. HB11690.
- 30 10. The antibody of Claim 9 which activates an erythropoietin receptor.
11. The antibody of Claim 9 wherein the erythropoietin receptor is a human erythropoietin receptor.
- 35

- 46 -

12. The antibody of Claim 9 which is a monoclonal antibody.

5 13. The antibody of Claim 9 which is a humanized antibody.

14. The antibody of Claim 9 having a detectable label.

10 15. A hybridoma cell line capable of producing the monoclonal antibody of Claim 12.

16. An antibody produced by the hybridoma cell line ATCC No. HB11689 or ATCC No. HB11690.

15 17. Hybridoma cell line ATCC No. HB11689 or ATCC No. HB11690.

18. A method of detecting in a biological sample an erythropoietin receptor which is capable of being activated, the method comprising the steps of:

(a) contacting the sample with the antibody of Claims 1 or 9;

(b) detecting the activation of the receptor by the antibody,

25 thereby determining the presence of an erythropoietin receptor which is capable of being activated.

30 19. A kit for detecting in a biological sample an erythropoietin receptor which is capable of being activated comprising the antibody of Claims 1 or 9.

20. A method of modulating the endogenous activity of an erythropoietin receptor in a mammal comprising 35 administering an amount of the antibody of Claims 1 or 9 effective to modulate the activity of the receptor.

- 47 -

21. The method of Claim 20 wherein the modulation of the erythropoietin receptor activity regulates proliferation or differentiation of erythroid progenitor cells.
- 5 22. A method of treating anemia in a patient comprising administering a therapeutically effective amount of the antibody of Claims 1 or 9.
- 10 23. A pharmaceutical composition comprising a therapeutically effective amount of the antibody of Claims 1 or 9 in a pharmaceutically acceptable adjuvant.
- 15 24. The composition of Claim 23 wherein the antibody is a monoclonal antibody.
25. The composition of Claim 24 wherein the antibody is a humanized antibody.
- 20 26. The composition of Claim 24 wherein the antibody is a human antibody.

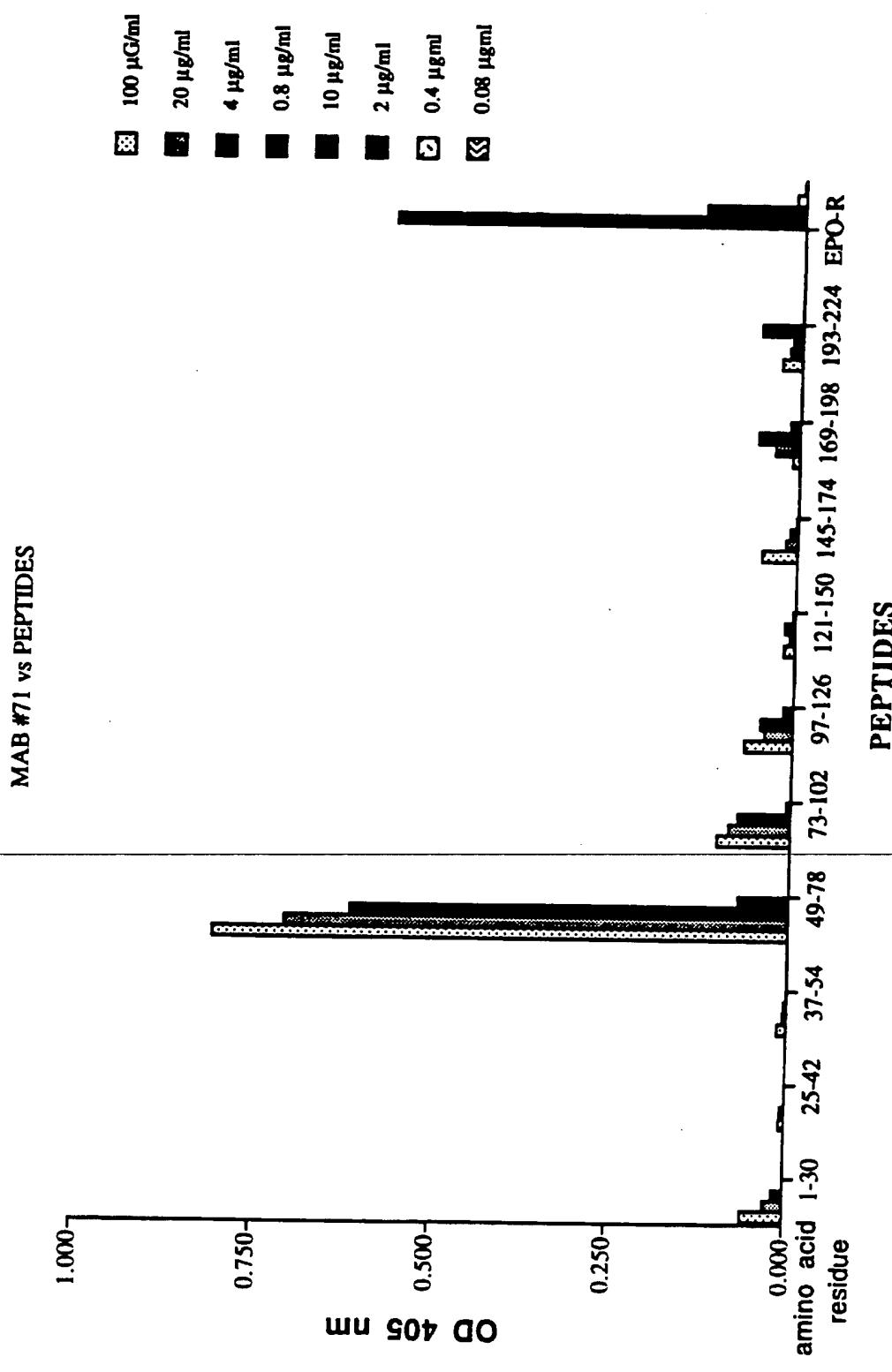


FIGURE 1

FIGURE 2

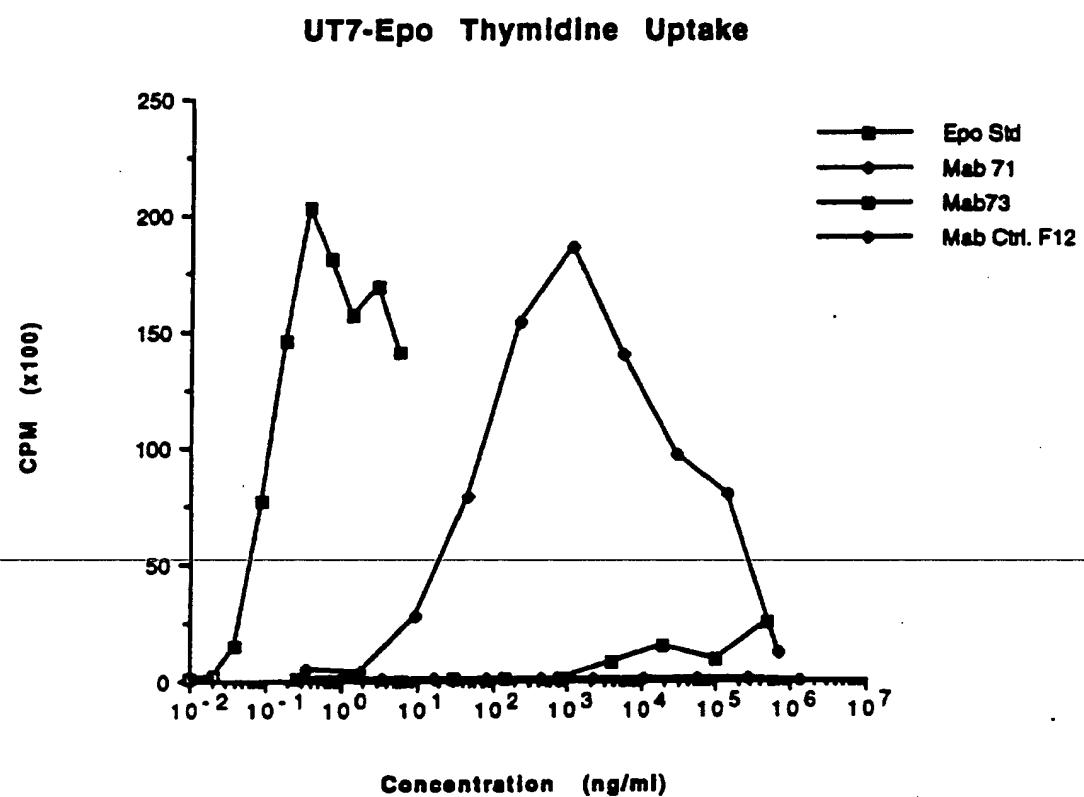


FIGURE 3

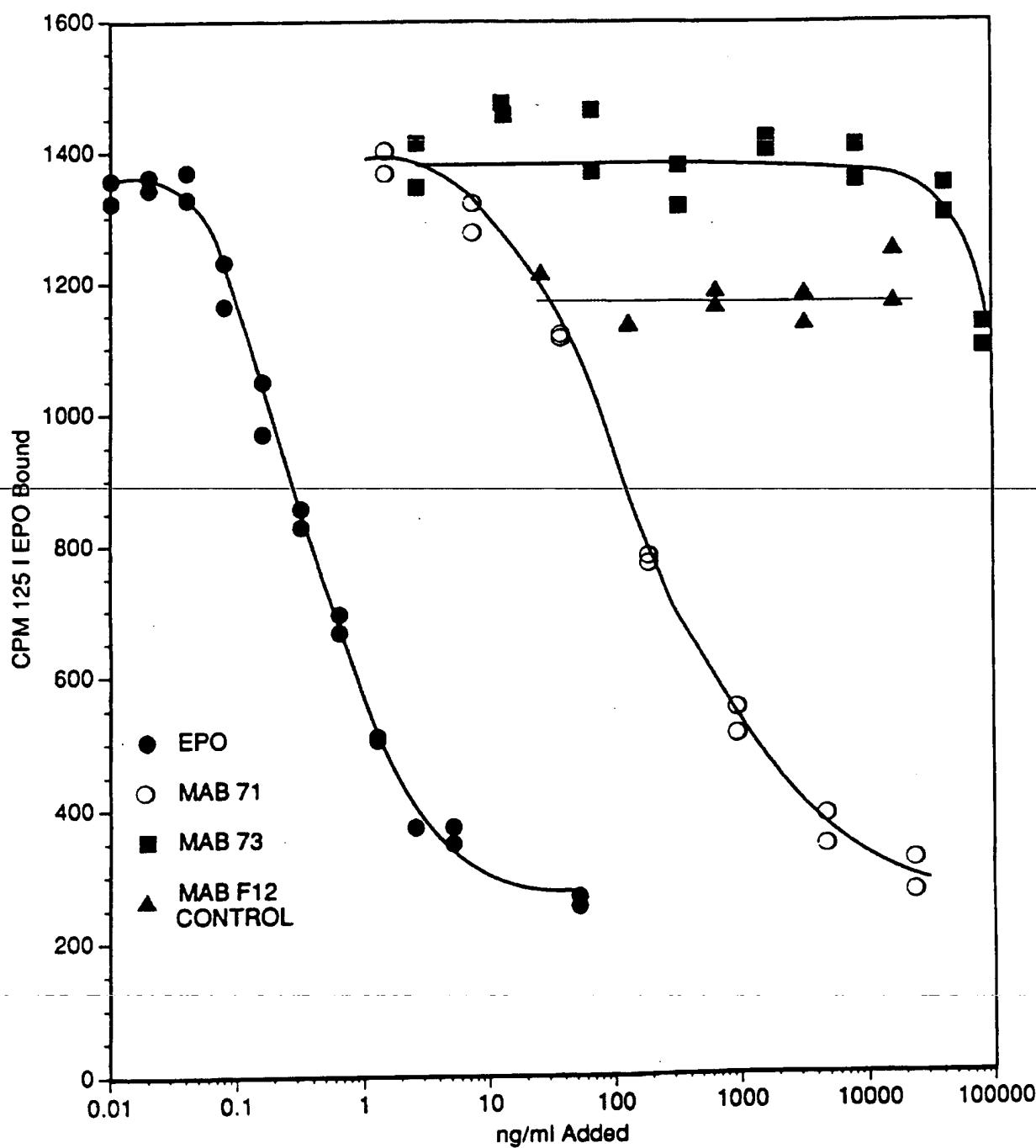
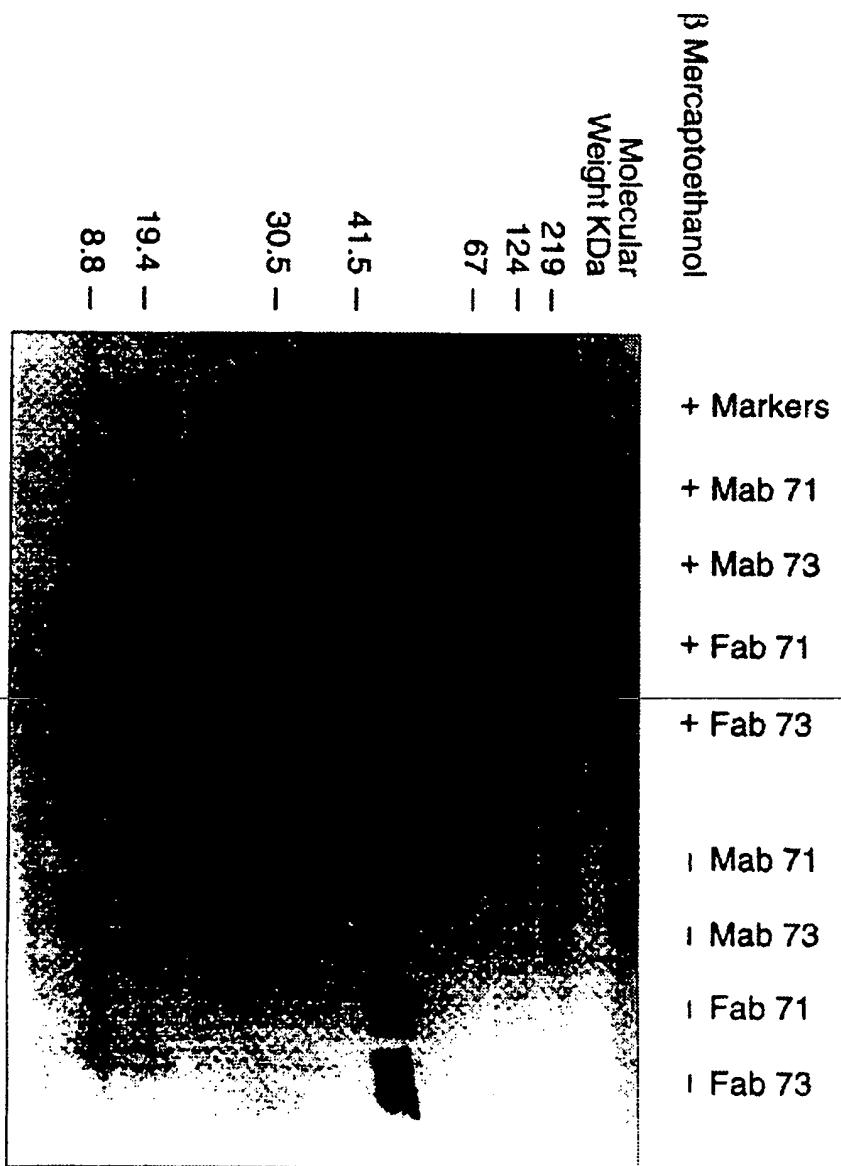
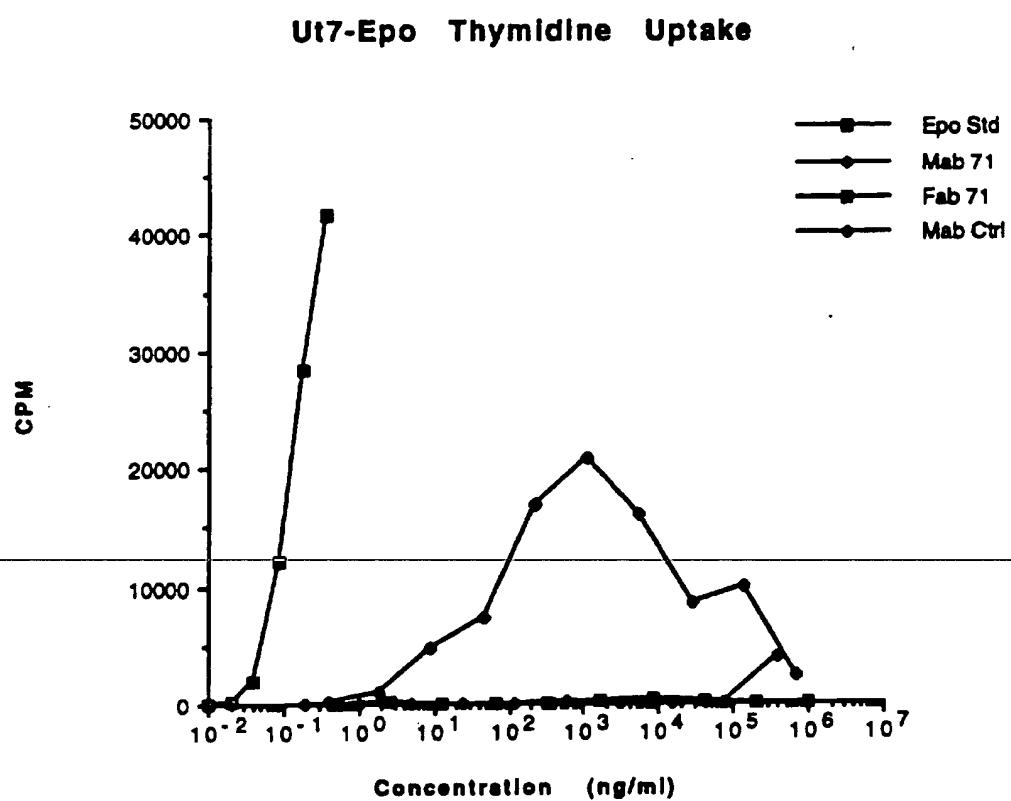
INHIBITION OF  $^{125}\text{I}$  EPO BINDING TO OCIM1 CELLS

FIGURE 4

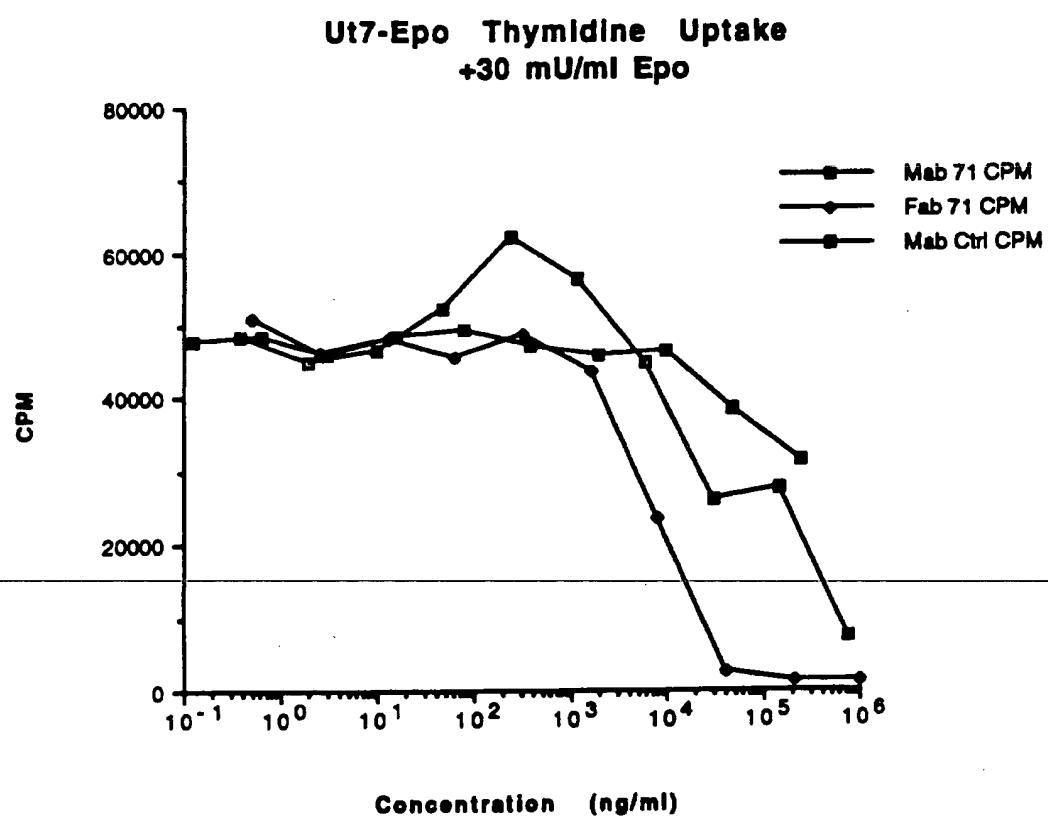


5/8

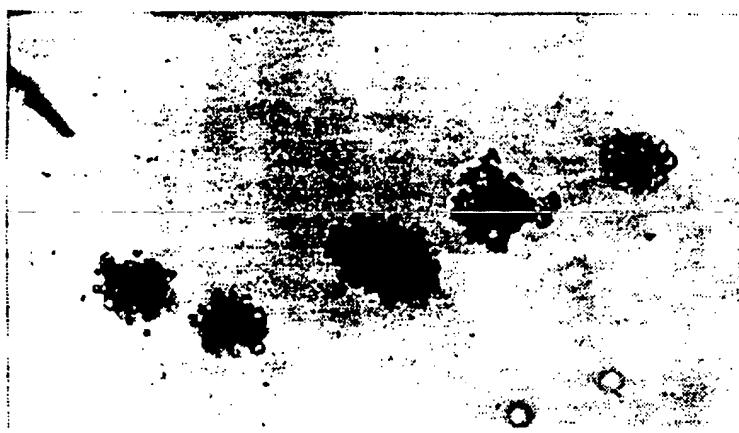
FIGURE 5



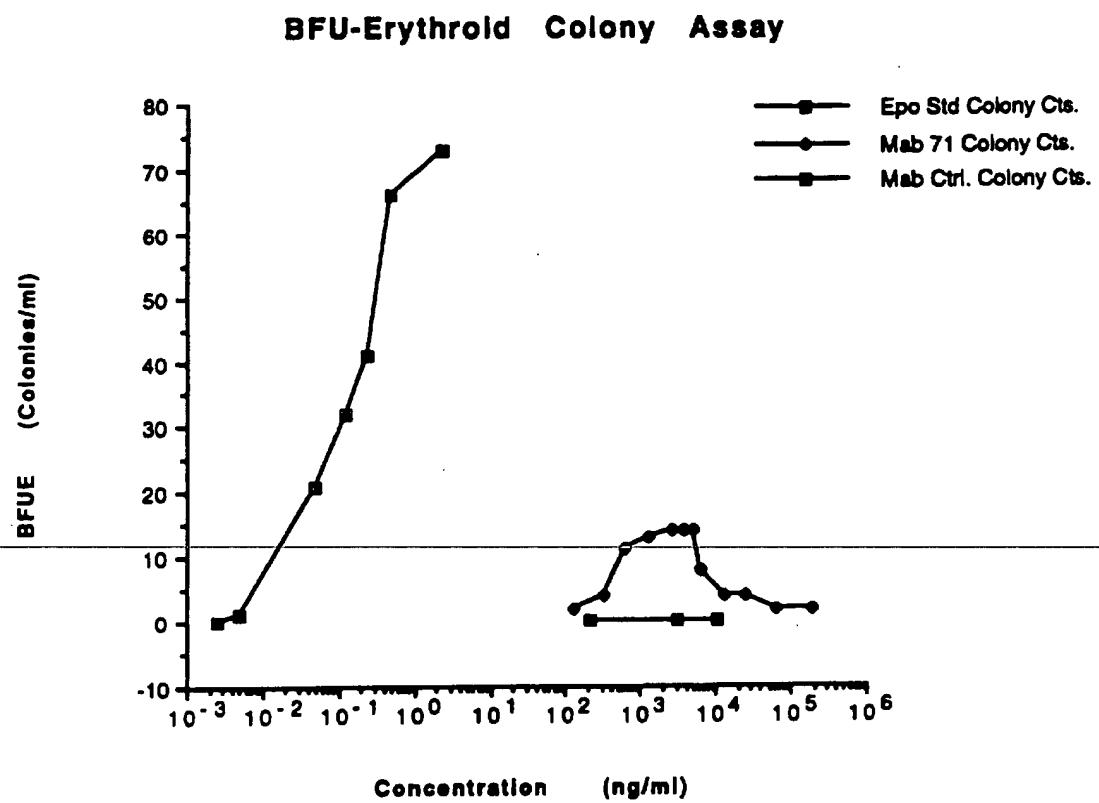
— FIGURE 6 —



— FIGURE 7

**A****B****C**

— FIGURE 8 —



# INTERNATIONAL SEARCH REPORT

Int'l Application No  
PCT/US 95/09458

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 6 C07K16/28 C12N5/20 G01N33/577 G01N33/68 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C07K C12N G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,90 08822 (GENETICS INSTITUTE INC. & WHITEHEAD INSTITUTE) 9 August 1990 cited in the application see page 12, line 4 - line 27 see claims ----	1-26
A	WO,A,92 21029 (GENENTECH INC.) 26 November 1992 see page 27, line 18 - page 28, line 17 see examples 8,10 see claims 13-15 ----	1-26
	----	

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

\* Special categories of cited documents :

- 'A' document defining the general state of the art which is not considered to be of particular relevance
- 'E' earlier document but published on or after the international filing date
- 'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- 'O' document referring to an oral disclosure, use, exhibition or other means
- 'P' document published prior to the international filing date but later than the priority date claimed

'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

'&' document member of the same patent family

1	Date of the actual completion of the international search  7 November 1995	Date of mailing of the international search report  30.11.95
	Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (- 31-70) 340-2040, Tx. 31 651 epo nl. Fax (- 31-70) 340-3016	Authorized officer  Nooij, F

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 95/09458

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ENDOCRINOLOGY, vol. 126, no. 3, March 1990 USA, pages 1318-1326, B. DATTATREYAMURTY ET AL. 'Polyclonal antibodies against follitropin (FSH) receptor interfere with hormone binding, but mimic the effects of FSH.' see abstract ---	1-26
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 91, no. 9, 26 April 1994 WASHINGTON DC, USA, pages 3974-3978, A. ANAGNOSTOU ET AL. 'Erythropoietin receptor mRNA expression in human endothelial cells.' see page 3975, left column, line 32 - line 36 ---	1-26
A	BLOOD, vol. 82, no. 10 suppl. 1, 15 November 1993 NEW YORK NY, USA, page 197a M. FISHER ET AL. 'Anti-erythropoietin receptor monoclonal antibodies distinguish EPO-dependent and EPO-independent erythroid progenitors in polycythemia vera.' cited in the application see abstract 774 ---	1-26
A	BLOOD, vol. 82, no. 1, 1 July 1993 NEW YORK NY, USA, pages 46-52, A. D'ANDREA ET AL. 'Anti-erythropoietin receptor (EPO-R) monoclonal antibodies inhibit erythropoietin binding and neutralize bioreactivity.' cited in the application see the whole document -----	1-26

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/09458

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: 20-22

because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 20-22 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2.

Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3.

Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**

International Application No

PCT/US 95/09458

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9008822	09-08-90	US-A-	5378808	03-01-95
		US-A-	5278065	11-01-94
WO-A-9221029	26-11-92	EP-A-	0586549	16-03-94
		JP-T-	6507715	01-09-94